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Cytosolic chaperones influence the fate of a toxin dislocated from the endoplasmic reticulum

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The plant cytotoxin ricin enters target mammalian cells by receptor-mediated endocytosis and undergoes retrograde transport to the endoplasmic reticulum (ER). Here, its catalytic A chain (RTA) is reductively separated from the cell-binding B chain, and free RTA enters the cytosol where it inactivates ribosomes. Cytosolic entry requires unfolding of RTA and dislocation across the ER membrane such that it arrives in the cytosol in a vulnerable, nonnative conformation. Clearly, for such a dislocated toxin to become active, it must avoid degradation and fold to a catalytic conformation. Here, we show that, *in vitro*, Hsc70 prevents aggregation of heat-treated RTA, and that RTA catalytic activity is recovered after chaperone treatment. A combination of pharmacological inhibition and cochaperone expression reveals that, *in vivo*, cytosolic RTA is scrutinized sequentially by the Hsc70 and Hsp90 cytosolic chaperone machineries, and that its eventual fate is determined by the balance of activities of cochaperones that regulate Hsc70 and Hsp90 functions. Cytotoxic activity follows Hsc70-mediated escape of RTA from an otherwise destructive pathway facilitated by Hsp90. We demonstrate a role for cytosolic chaperones, proteins typically associated with folding nascent proteins, assembling multimolecular protein complexes and degrading cytosolic and stalled, cotranslocational clients, in a toxin triage, in which both toxin folding and degradation are initiated from chaperone-bound states.

Hsc70 | Hsp90 | ricin

Endoplasmic-reticulum (ER) associated protein degradation (ERAD) comprises coordinated disposal systems that recognize and remove misfolded and unassembled proteins in the ER, dislocating them across the ER membrane to the cytosol for proteasomal destruction. Degradation is normally facilitated by polyubiquitylation, usually on internal lysyl residues of the target protein. Both membrane-bound and soluble ER proteins can be disposed of by ERAD (1, 2).

The plant cytotoxin ricin traffics to the ER lumen of mammalian cells where it is reduced to its RTA and RTB subunits before RTA dislocation (3). RTA does not penetrate the ER membrane directly; instead it exploits pre-existing protein-conducting channels as a nonnative species, mimicking ER proteins dispatched via ERAD (4, 5). Thus, it enters the cytosol in a form susceptible to proteolysis or aggregation. A proportion must evade these fates to gain a catalytic conformation that depurinates target ribosomes, stopping protein synthesis. The paucity of lysine residues in RTA may facilitate uncoupling from ERAD by reducing the potential for polyubiquitylation, thereby hampering proteasomal degradation (6). This, in turn, may provide opportunities for spontaneous or chaperone-assisted folding not normally sanctioned for ERAD substrates.

We show an interaction of RTA with the cytosolic heat shock (cognate) protein Hsc70. From the chaperone-bound state, nonnative cytosolic RTA can achieve a catalytic conformation or can be inactivated. Its ultimate fate depends on the activities of cochaperones that regulate Hsc70.

Results

Inhibition of Hsc70 Protects HeLa Cells from Ricin. Ricin binds exposed galactosyl residues, opportunistically exploiting a huge number of surface glycoproteins (3). Consequently, trafficking pathways are diverse with <5% of the cell-surface bound toxin reaching the TGN (7). Of this, only a tiny proportion reaches the ER (8) and subsequently the cytosol. RTA is not modified in transit, so the toxic cytosolic fraction cannot be distinguished from the overwhelming noncytotoxic majority at the cell surface and in the endomembrane system by immunoblotting, cellular fractionation, indirect immunofluorescence, or coimmunoprecipitation approaches. However, ricin cytotoxicity correlates strongly with ribosome depurination, so it can be used as a measure of the relative amount of native RTA in the cytosol (9).

Deoxyspergualin (DSG) alters the ATPase activity of the cytosolic heat-shock (cognate) protein Hsc70 *in vitro*, and *in vivo* permits expression of functional cystic fibrosis transmembrane conductance regulator (CFTR) in cells expressing mutant $\Delta F508$ CFTR, presumably by inhibiting interactions with Hsc70 chaperone complexes that target it to proteasomes (10, 11). When DSG was added with ricin to HeLa cells, the cells became ~3-fold more resistant to toxin (Fig. 1*A* and *B*), suggesting that an increased proportion of toxin remained inactive. DSG alone had no effect on protein synthesis (Fig. 1*C*). The ER Hsp70 counterpart BiP is not affected by DSG (12), suggesting that protection occurred in the cytosol. After ricin challenge, a distinct lag before the onset of cytotoxicity that represents trafficking time from cell surface to the first destruction of ribosomes (13) is unchanged by DSG treatment (Table 1), confirming that the effect of DSG occurs in the cytosol, and not by interfering with toxin delivery. Because unfolding is necessary to render RTA competent for dislocation (5), then folding must be required for catalytic activity in the cytosol, and this appears to involve Hsc70. In contrast, DSG had no effect on the potency of diphtheria toxin (DTx, Fig. 1*A* and *B*), which enters the cytosol from acidified endosomes, refolding spontaneously in the cytosol (14).

Inhibition of Hsp90 Sensitizes Cells to Ricin. DSG also interacts with the cytosolic chaperone Hsp90 (10). To clarify its most upstream target, we used geldanamycin (GA) and radicicol (RA), competitive inhibitors of the Hsp90 ATP-binding site (15). Treatment with 1 μ M GA or RA resulted in a ~1.5- to 2-fold sensitization of cells to ricin (Fig. 2*A* and *B*), without altering toxin trafficking times (Table 1). Treatments with GA or RA alone have little, if any, effect on protein synthesis (Fig. 2*C*).

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The authors declare no conflict of interest.

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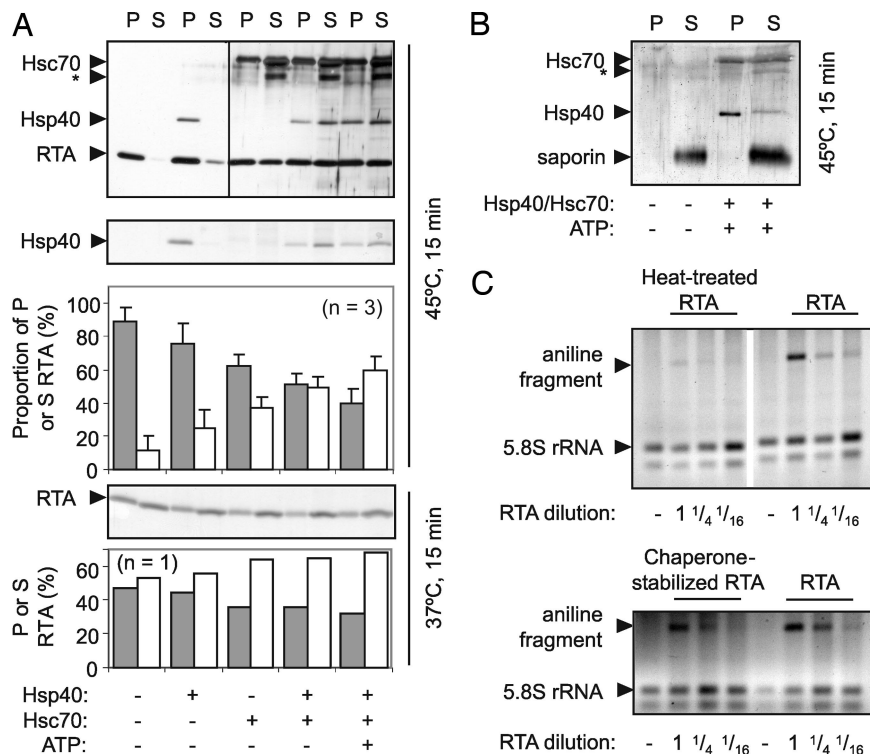


Fig. 3. *In vitro* interactions of RTA and Hsp40 and Hsc70 chaperones. (A) RTA (500 ng) was incubated at the indicated temperatures for 15 min in 20 μ l of 20 mM Mops pH 7.2, 100 mM KCl, in the presence or absence of Hsp40, Hsc70, or ATP (shown below the panels). Aggregated (P) and soluble (S) fractions were separated by centrifugation, heated in reducing SDS sample buffer, and analyzed by SDS/PAGE and subsequent silver staining. *, proteolytic fragment of Hsc70 lacking the C-terminal regulatory domain. Proportions (%) of aggregated (gray) and soluble (white) RTA are shown. (Bars, \pm 1 S.D.) (B) Reaction mixtures where RTA was replaced by saporin were treated as in (A). (C) Dilutions of the soluble fractions from 375 ng of heat-treated RTA (upper panel), 375 ng of chaperone-stabilized RTA (lower panel), and 375 ng of native RTA were added to yeast ribosomes for 2 h at 30°C. After cleavage of any depurinated 28S rRNA with acetic-aniline, rRNAs were extracted, electrophoresed in denaturing conditions (1.2% agarose, 50% formamide), and gels were stained with ethidium bromide before quantifying (22).

When RTA was heated for 15 min with or without chaperones at 37°C, the chaperones had a similar although less pronounced effect (Fig. 3A, lower panel, lower graph). Saporin has a largely indistinguishable tertiary structure relative to RTA with identical, superimposable active site residues and has identical activity against ribosomes (20), but it lacks the C-terminal hydrophobic stretch that in RTA has been implicated in membrane interactions preceding dislocation (21). Saporin showed no thermal instability (Fig. 3B) after heating at 45°C. This result contrasts with the instability of RTA and its recognition by Hsp40/Hsc70 even at 37°C, and suggests that interactions with Hsc70 might be a normal feature of folded RTA in the cytosol.

To investigate whether chaperone-treated RTA had catalytic activity, dilutions of the soluble fractions from heat-treated RTA and chaperone-stabilized RTA were added to yeast ribosomes, which were subsequently treated with acidified aniline. Correctly folded RTA specifically depurinates yeast 26S rRNA, and aniline treatment cleaves the phosphodiester bond at the depurination site, releasing a small diagnostic fragment of RNA (22). As expected, a small amount of RTA activity remained after heat-treatment, but almost full activity was recovered from chaperone-stabilized RTA (Fig. 3C). We conclude that the major proportion of denatured RTA with chaperone-mediated solubility is, or can become, competent to gain catalytic activity.

Hsc70 Cochaperone Activity Determines the Fate of Dislocated RTA *in Vivo*. Having established that RTA interacts with Hsc70 *in vitro* and that catalytic activity can be recovered from the chaperone-

bound state, we examined the effects of modulating Hsc70 cochaperone activities *in vivo*.

The dual cochaperone Hsc70-Hsp90 organizing protein (Hop) recruits Hsp90 to pre-existing Hsc70-client complexes, transferring client proteins from Hsc70 to Hsp90 (23). Transient overexpression of Hop protected cells from ricin challenge (Fig. 4A and C), suggesting that increased links between Hsc70 and Hsp90 lead to increased toxin turnover, consistent with the sensitizing effects of GA/RA that block entry into the Hsp90 cycle. Thus, sequential interaction with Hsc70 and Hsp90 directs cytosolic RTA toward net inactivation.

BCL2-associated athanogene protein (BAG-1) isoforms are nucleotide exchange factors that stimulate release of Hsc70-bound clients and bear a ubiquitin-like (ubl) motif that interacts with the proteasome (24). Transient overexpression of BAG-1 protected cells against ricin (Fig. 4B and D). Thus, BAG-1 may stimulate release of Hsc70-bound RTA on a path toward inactivation, consistent with the increased sensitivity of cells to ricin reported after inhibition of proteasome activity (25).

Both Hsc70- and Hsp90- complexes can become sorting machines that promote client destruction. C terminus of Hsp70-interacting protein (CHIP) is an E3 ubiquitin ligase that interacts with Hsc70 and Hsp90, and with its partner ubiquitin-conjugating enzymes initiates proteasomal sorting by ubiquitylating chaperone-bound substrates. Transient overexpression of CHIP resulted in enhanced inactivation of RTA, seen as increased resistance of cells to ricin (Fig. 4B and E).

To investigate whether Hsc70 can also promote toxin activity *in vivo*, we overexpressed Hsp70-interacting protein (Hip), a Hsc70 cochaperone, which stabilizes ADP-bound Hsc70, in-

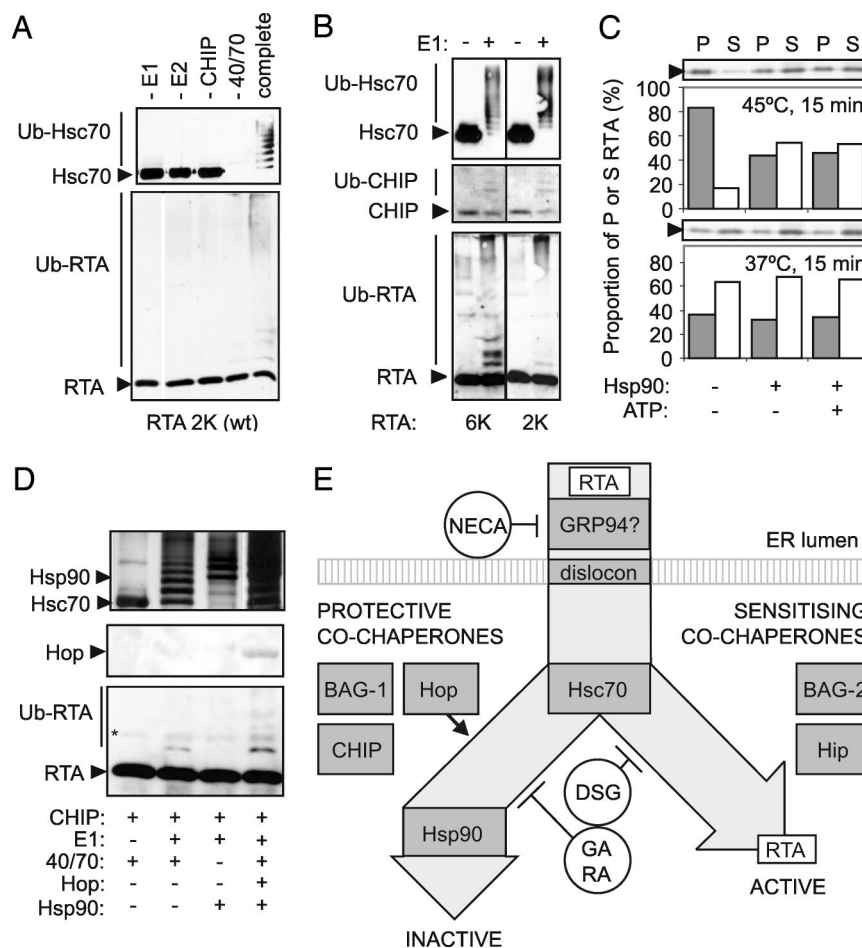


Fig. 5. RTA is a substrate for CHIP. (A) RTA was added to a reaction mixture (complete), which can recapitulate CHIP activity (28), and to mixtures lacking E1 ubiquitin activating enzyme, E2 (UbcH5 ubiquitin conjugating enzyme), CHIP, or Hsp40/Hsc70 (40/70) as indicated. After heating (45°C, 10 min), reactions were activated by addition of 5 mM MgCl₂, 10 mM DTT, and 5 mM ATP, incubated (2 h, 30°C), and products were identified by reducing SDS/PAGE and immunoblotting for Hsc70 and RTA. (B) Addition of E1 to a reaction mixture containing RTA, Hsp40, Hsc70, E2, CHIP, and ubiquitin as in A, and incubation at 37°C for 2 h results in CHIP-mediated ubiquitylation of Hsc70 (upper panel, Ub-Hsc70), CHIP (middle panel, Ub-CHIP), and both RTA 6K and RTA (lower panel, Ub-RTA) as revealed by immunoblots. (C) RTA (500 ng) was incubated (45°C or 37°C, 15 min) in the presence or absence of Hsp90 or ATP (shown below the panels). Aggregated (P) and soluble (S) fractions were separated by centrifugation, heated in reducing SDS sample buffer, and analyzed by SDS/PAGE and subsequent silver staining. Proportions (%) of aggregated (gray) and soluble (white) RTA are shown. (D) RTA was added to CHIP recapitulation mixtures as in A, but containing Hsp40 and Hsp70 (40/70), HOP, and Hsp90 as indicated below the panels, incubated (2 h, 37°C), and products were identified by reducing SDS/PAGE and immunoblotting for HOP and RTA, and by silver staining for Hsc70 and Hsp90. *, cross-reacting contaminant. (E) Proposed cytosolic triage of dislocated RTA.

of Hsc70 supporting the folding of RTA directly cannot yet be discounted.

Folding of heat-denatured RTA *in vitro* is favored in the presence of ribosomes, implicating the toxin's substrate in its own activation (19). It remains to be seen whether human homologues of ribosome-tethered chaperones that assist folding of nascent proteins (37, 38) also contribute to posttranslational folding of cytosolic RTA and how significant a role they have relative to the soluble chaperone complexes.

Materials and Methods

Cytotoxicity Measurements. HeLa cell responses to 4-h challenges with graded doses of ricin or diphtheria toxin (DTx) were measured as previously described (3). For pharmacological studies, cells were treated coevally with graded doses of toxin in medium containing carrier or solvent vehicle (control) and with toxin dilutions in medium containing both carrier/vehicle and pharmacological agent. Each cytotoxicity curve was normalized to controls not treated with toxin, but containing agent or vehicle as appropriate, so any effects of agent alone on protein synthesis were accounted for. Toxin trafficking times from cell surface to first destruction of ribosomes were measured as previously described (13). DSG was supplied by Worldwide Clinical Development, Nippon Kayaku Co., Ltd., 31-12, Shimo 3-chome, Kita-ku, Tokyo 115-0042, Japan.

Overexpression Studies. HeLa cells were transfected with vectors expressing cochaperones, using previously published conditions (3). Two days posttransfection, cells were seeded into 96-well plates and grown overnight for cytotoxicity studies. Overexpression of cochaperones was confirmed by using FLAG-tagged versions of these proteins, and immunoblotting using an anti-FLAG antibody (data not shown).

In Vitro Ubiquitylation, Aggregation, and RTA Activity Studies. Recombinant RTA was added to reaction mixtures containing 0.1 μ M E1 ubiquitin activating enzyme, 0.3 μ M Hsp40, 3 μ M Hsc70, 3 μ M HOP, 3 μ M Hsp90, 4 μ M UbcH5 (E2 conjugating enzyme), 3 μ M CHIP, and 2 mg·ml⁻¹ ubiquitin in 20 mM Mops, pH 7.2, 100 mM KCl, 5 mM MgCl₂, 5 mM ATP, 10 mM DTT buffer as previously described (28) and to reaction mixtures lacking components as appropriate. After incubation (2 h) at 30°C or 37°C as appropriate, products were identified by reducing SDS/PAGE and immunoblotting. When required, reaction mixes lacking ATP and MgCl₂ were heated to 45°C for 10 min followed by cooling to the required reaction temperature and activation of the reaction by addition of ATP and MgCl₂. For aggregation studies, reaction mixtures lacking E1, E2, CHIP, and Ub were used, and RTA was heated to 37°C or 45°C in the presence or absence of Hsp40, Hsc70, Hsp90, and ATP. Aggregated and soluble fractions were separated by centrifugation (16,000 \times g, 10 min), solubilized in reducing SDS sample buffer, and analyzed by SDS/PAGE and silver staining. Catalytic activity of RTA from soluble fractions was assayed by quantifying the RNA

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